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(54) Title: METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

(57) Abstract

Inhibitors and activitors of a protein whose expression affects a phenotypic characteristic of the cell, especially a cultural or morphological characteristic, are identified by their more pronounced effect on cells producing higher, usually non-naturally occurring, levels of the protein, than on cells producing little or none of the protein. In a preferred assay, the effect is observable with the naked eye. By this method, tamoxifen is identified as an inhibitor of PKC activity in cell culture.

METHOD OF SCREENING FOR PROTEIN INHIBITORS AND ACTIVATORS

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to a general screening method for the discovery and identification of both inhibitors and activators of enzymes, receptors, and other proteins. In particular, it is concerned with a method of screening for substances which specifically inhibit or activate a particular protein affecting the cultural or morphological characteristics of the cell expressing the protein, especially in a manner apparent to the naked eye.

Information Disclosure Statement

A number of assay systems are currently in use for the discovery of new modulators of cell growth, and in particular, in the search for new anti-cancer drugs which are specifically toxic to cancer cells but not to normal cells. A variety of changes may be scored for, but the most common ones are reversion of the transformed phenotype, significant changes in cell morphology, or cytotoxicity. The assays include: (1) in vitro cytotoxicity assays; (2) soft agar colony formation assays; (3) in vitro anti-microbial assays; and (4) assays which detect changes in cellular morphology.

In vitro cytotoxicity assays involve the measurement of cellular parameters which are indicative of inhibition of cellular growth or cytotoxicity. These include, for example, the measurement of the inhibition of certain cellular metabolic pathways in

response to treatment with cytotoxic agents. The papers by Von Hoff, et al. (1985), and Catino, et al. (1985) describe typical methods which use this technique. These methods are somewhat complex technically, and require the use of radioactive tracers in some cases. Furthermore, the results are non-specific since any agent which alters the growth properties of cells will score positively in these assay systems.

Agents have also been tested for their ability to inhibit transformed (cancerous) cells from growing in soft agar. This method is based upon the finding by Freedman and Shin (1974) that the formation of colonies of cells in soft agar is the _in vitro_ test which shows the highest correlation in predicting whether the cells will be tumorigenic in an experimental animal. This method is relatively simple to perform since colony growth will, after two or more weeks, generally be large enough to be seen with the naked eye. Scoring the final results, therefore, can be performed either by a technician without extensive training in tissue culture, or, as we describe in the current application, by an automated absorbance detection system. In its present form, however, this method is also non-specific for the same reasons as described above. In other words, any agent which inhibits cellular growth in any way will scores positively in this assay system as it is currently used, whether or not it inhibits the protein of interest.

in vitro anti-microbial assays involve the use of bacterial or yeast strains which are used as test organisms for screening for agents with generalized growth inhibitory properties (also described in Catino,

et al., 1985). In this method, the bacterial or yeast strain is grown on standard media plates and potential agents are applied to various spots on the plates. If an agent has growth inhibitory properties, a clear zone results at the site of its application on the plate, resulting from the inability of the test strain to grow in the area. This method is rapid and can be performed by a technician without extensive training in tissue culture techniques, but the results are generally non-specific because agents which are effective against bacterial or yeast strains are frequently less effective (or completely ineffective) in modulating the growth of mammalian cells, as shown in the paper by Catino et al. (1985).

Still other screening systems depend upon a morphologic alteration of the test cells by exposure to the potential agents in order to determine the effectiveness of a given agent. This method is currently the most effective one for developing specific agents which interact with a given protein or alter a specific cellular property, as evidenced by the representative paper by Uehara, et. al. (1985). However, these screening systems are the most difficult ones to apply in practice, since the morphologic effect of each individual agent on the test cells must be studied under the microscope. Hence this method requires extensive observations of the cells by a trained scientist.

SUMMARY OF THE INVENTION

The Method presented in detail in this application combines the rapidity and ease of performance of the soft agar assay with a specificity for detecting an

active agent exceeding that of the morphology assay. In brief, the method which we describe herein involves the generation of a cell line purposefully engineered to detect both stimulatory and inhibitory agents which are absolutely specific for any given protein which affects the cultural or morphological characteristics of the cell.

The basis for this invention is my observation that if a protein (the "protein of interest", or POI) which is involved in some manner in cellular growth control is overproduced in cells, then pharmacologic agents which can activate or inhibit the POI can result in altered growth properties of the cells.

The sensitivity of the cells is dependent on their production of the POI, a phenomenon referred to herein as a "graded cellular response" to the pharmacologically active agent.

The present invention provides a rapid, yet powerful screening system for the discovery and identification of both inhibitors and activators of proteins. The method may be applied to virtually any type of protein, including enzymes, receptors, DNA- or RNA-binding proteins, or others which are directly or indirectly involved in regulating cellular growth.

The method involves the insertion of a DNA (or cDNA) sequence encoding the Protein Of Interest (POI) into an appropriate vector and the generation of cell lines which contain either (1) the expression vector alone ("control" cell lines) or (2) the expression vector containing the inserted DNA (or cDNA) sequence encoding the POI ("test" cell lines). Using the

appropriate vector system, recipient cell lines, and growth conditions, test cell lines can thus be generated which stably overproduce the corresponding POI. Under the appropriate growth conditions, these cell lines will exhibit a "graded cellular response" to activators or inhibitors of the POI. A screening system can thus be set up whereby the control and test cell lines are propagated in defined growth conditions in tissue culture dishes (or even in experimental animals) and large numbers of compounds (or crude substances which may contain active compounds) can be screened for their effects on the POI.

Substances which inhibit or activate the POI may affect characteristics such as growth rate, tumorigenic potential, anti-tumorigenic potential, anti-metastatic potential, cell morphology, antigen expression, and/or anchorage-independent growth capability. Substances which specifically inhibit or inactivate the POI may be distinguished from substances which affect cell morphology or growth by other mechanisms in that they will have a greater effect on the test lines than on the control lines.

The system has been tested using several cDNA sequences and several recipient cell lines, and can be easily automated.

The appended claims are hereby incorporated by reference as an enumeration of the preferred embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the full-length cDNA sequence, and the deduced amino acid sequence, of one of several forms of PKC which has previously been isolated (cDNA clone RP58), and whose partial sequence has been reported (Housey, et al., 1987). It corresponds to PKCbeta1 according to the nomenclature of Ono, et al. (1987). The deduced amino acid sequence begins with the first in-frame methionine codon at nucleotide position 91 and encodes a 671 amino acid protein with a predicted molecular weight of 76.8 kd. A consensus polyadenylation signal is underlined.

Figure 1B shows the retrovirus-derived cDNA expression vector, developed in this laboratory, which was used for the present studies. The full-length RP58 cDNA encoding PKCbeta1 (shown in 1A) was cloned into the Eco RI site of plasmid pMV7. The shaded region represents the coding sequence. "E" and "P" designate Eco RI and Pst I restriction sites, respectively. The indicated sizes between restriction sites in the RP58 cDNA are given in kilobases. "LTR" designates the 5′ (left) and 3′ (right) long terminal repeats of Moloney murine leukemia virus, and "TK-neo" designates the promoter region of the HSV thymidine kinase gene linked to the 5′ end of the bacterial neomycin phosphotransferase (neo) gene.

Figure 1-C outlines in schematic form the overall strategy used to generate cell lines stably overproducing PKC.

Figure 2. Purification and Autophosphorylation of PKC. PKC activity from each cell line was purified and subjected to reaction conditions favoring autophosphorylation of PKC. Following the

autophosphorylation reaction, protein samples were separated by discontinuous polyacrylamide gel electrophoresis. In the lanes bearing odd numbers the reaction mixtures contained 1 mM Ca2+ and phosphatidylserine to activate PKC, and in the lanes bearing even numbers the reaction mixtures contained 1 mM EGTA, 100 ng/ml TPA, and phosphatidylserine. The numbers in the left margin indicate the sizes of molecular weight markers, in kd. Arrows indicate the position of the 75 kd autophosphorylated PKC.

Figure 3. Northern Blot Hybridization Analyses. Poly A+ RNA was isolated from the indicated cell lines and separated by electrophoresis on 6% formaldehyde/1% agarose gels, blotted onto nylon membrane and hybridized with the 32P-labelled full-length RP58 cDNA probe, as previously described (Housey, et. al., 1987). The numbers in the right margin indicate the sizes in kb of the RNA markers. The R6-PKC4 sample displayed a very weak 4.8 kb band on the original autoradiograph.

Figure 4. Morphologic Responses of the Cell Lines to Phorbol Ester Treatment. Nearly confluent cultures of the three indicated cell lines were exposed to 100 ng/ml TPA in 0.1 % dimethylsulfoxide (DMSO) solvent ("+TPA") or 0.1% DMSO alone ("-TPA"), in DMEM plus 10% CS. Photographs were taken 24 hours later (Panels A and D) and 48 hours later (Panels B and E). Fresh medium plus or minus TPA was then added and photographs were then taken an additional 24 hours later (Panels C and F). (Magnification : 100X).

Figure 5. Growth Curves of Control and PKC-Overproducing Cell Lines. The indicated cell lines were seeded at 1 x 10^4 per 6 cm plate in DMEM plus 10%

CS, in the presence ("+TPA") and absence ("-TPA") of 100 ng/ml TPA. Cell numbers were determined in replicate plates during the subsequent 11 day growth period. The values given indicate the means of triplicate determinations, which varied by less than 10%.

Figure 6. Post-confluence Foci Formation. Control R6-C2 cells (Panel A) and R6-PKC3 cells (Panels B and C) were grown to confluence and then maintained for an additional 28 days in DMEM plus 10% CS (without TPA) , with the addition of fresh medium every 3 days. Photographs were taken at the end of the 28 day period. Magnification : 40 X.

Figure 7. Growth in Soft Agar. Cell lines R6-C1, R6-PKC3, and R6-PKC5 were seeded into 60 mm petri dishes in 0.3% agar containing DMEM plus 20% FBS and 50 lg/ml G418, plus or minus 100 ng/ml TPA. Photographs were taken after 21 days of growth.A) R6-C1 + TPA (low-power field) B) R6-C1 + TPA (medium-power field)C) R6-PKC3 D) R6-PKC3 + TPA E) R6-PKC5 F) R6-PKC5 + TPAFor additional details see Experimental Procedures. (Magnification : 100 X in panel B; 40 X in all other panels).

DETAILED DESCRIPTION OF THE INVENTION

The present method is intended for use in identifying potential chemical inhibitors or activators of enzymes, receptors, or any proteins which have effects upon cell phenotype. This method requires two cell lines, preferably alike except for their expression (production) of the protein of interest at different levels (and any further differences necessitated by that difference in expression). Inhibitors or activators are identified by their greater effect on the phenotype of the higher producing cell line.

Any phenotypic characteristic of the cell which is affected by expression of the protein of interest, other, of course, than the level of the protein itself, may be assayed. The phenotypic characteristic is preferably a "cultural" or "morphological" characteristic of the cell. For purposes of the appended claims, these terms are defined as follows:

Cultural characteristics include, but are not limited to the nutrients required for growth, the nutrients which, though not required for growth, markedly promote growth, the physical conditions (temperature, pH, gaseous environment, osmotic state, and anchorage dependence or independence) of the culture which affect growth, and the substances which inhibit growth or even kill the cells.

Morphological characteristics, but are not limited to include the size and shape of cells, their arrangements, cell differentiation, and subcellular structures.

Where the protein of interest is implicated in tumorigenesis or related phenomena,, the characteristic observed is preferably one related to cellular growth control, differentiation, de-differentiation, carcinogenic transformation, metastasis, tumorigenesis, or angiogenesis.

Phenotypic changes which are observable with the naked eye are of special interest. Changes in the ability of the cells to grow in an anchorage-independent manner, to grow in soft agar, to form foci in cell culture, and to take up selected stains are particularly appropriate phenomena for observation and comparison.

The higher producing cell line is preferably obtained by introducing a gene encoding the Protein of Interest (POI) into a host cell. The gene may be a one isolated from the genome of an organism, a cDNA prepared from an mRNA transcript isolated from an organism, or a synthetic duplicate of a naturally occurring gene. It may also have a sequence which does not occur exactly in nature, but rather corresponds to a mutation (single or multiple) of a naturally occurring sequence. No limitation is intended on the manner in which this mutated sequence is obtained. The gene is operably linked to a promoter of gene expression which is functional in the host, such that the corresponding Protein Of Interest (POI) is stably "overproduced" in the recipient cells to differing degrees. The promoter may be constitutive or inducible. By "overproduced", I mean that the POI is expressed at higher levels in the genetically manipulated cell line than in the original cell line.

This allows one to generate cell lines which contain (or secrete) from as little as a few fold to more than 100-fold elevated levels of the POI relative to the control cells.

Any method may be used to introduce the gene into the host cell, including transfection with a retroviral vector, direct transfection (e.g., mediated by calcium phosphate or DEAE-dextran), and electroporation. Preferably, a retroviral vector is used.

The host cells should exhibit a readily observable phenotypic change as a result of enhanced production of the POI. Preferably, this response should be proportional to the level of production of the POI. Finally, the cells should not spontaneously manifest the desired phenotypic change. For example, 3T3 cells form foci spontaneously. Among the preferred cell lines are Rat-6 fibroblasts, C3H10T 1/2 fibroblasts, and HL60. (HL60 is a human cell line that differentiates in response to PKC activation.) 3T3 cells may be used, but with the reservation stated above.

Generally speaking, it is preferable to maximize the ratio of production by the "overproducing" cell line to production by the "native" line. This is facilitated by selecting a host cell line which produces little or no POI, and introducing multiple gene copies and/or using high signal strength promoters.

The Rat 6 embryo fibroblast cell line is a variant of the rat embryo fibroblast cell line established by Freeman et. al., (1972) and isolated by Hsiao et al.,

1986. This cell line has an unusually flat morphology, even when maintained in culture at post-confluence for extended periods of time, displays anchorage dependent growth and, thus far, has not undergone spontaneous transformation. It was also ideal for these studies since it has a very low level of endogenous PKC activity and a low level of high affinity receptors for phorbol esters.

While my most preferred host cell line is the Rat-6 fibroblast cell line, I have tested this Method with other cell types, including the mouse NIH-3T3 cell line as well as the C3H 10T1/2 cell line. Tables 1(a) and (b) below show the representative specific activities of seven NIH-3T3 and six C3H-10T1/2 cell lines stably overproducing PKC. I have also recently performed the same experiments with the human HeLa cell line. In each case the resulting cell lines all exhibited growth properties qualitatively identical to those described for the PKC-expressing Rat-6 fibroblast cell lines. Therefore these results clearly demonstrate that many different types of cells can be employed in this method. The experimental procedures used to generate these cell lines were also identical to those used in connection with the Rat-6 cell line.

If a cell line otherwise suitable for use as a control cell line produces excessive POI, it is possible to inhibit this production by incorporation of a known inhibitor into the culture medium for both the control and test cell lines, thus achieving a more favorable ratio of production. Contrariwise, if the level of POI production by the test cell line is too low, a known activator may be incorporated into the culture media.

It is desirable, but not necessary, that a suspected inhibitor or activator be tested on both a control line and an overproducing line in parallel.

What we are looking for is a <u>increase</u> in the phenotypic change exhibited by the cell which becomes greater with increasing expression of the POI. We call this a "graded cellular response," and it is by this specialized response that we distinguish inhibitors or activators of the POI from agents that act upon other cell metabolites to effect a phenotypic change.

Thus, in a preferred embodiment, the cell lines are assayed for their relative levels of the POI, and their ability to grow in anchorage-independent systems (e.g., matrices such as soft agar or methocel), to form small "foci" (areas of dense groups of cells clustered together and growing on top of one another) in tissue culture dishes, to take up selected stains, or to bind an appropriately labeled antibody or other receptor for a cell surface epitope. In addition to exhibiting these growth control abnormalities, such cell lines will also be sensitive in their growth properties to chemical agents which are capable of binding to, or modifying the biological effects of, the POI.

The method is particularly unique in that it can be employed to search rapidly for EITHER activators OR inhibitors of a given POI, depending upon the need. The term "activators," as used herein, includes both substances necessary for the POI to become active in the first place, and substance which merely accentuate its activity. The term "inhibitors" includes both substance which reduce the activity of the POI and

these which nullify it altogether. When a POI has more than one possible activity. The inhibitor or activator may modulate any or all of its activities.

The use of this screening method to identify inhibitors or activators of enzymes is of special interest. In particular, I am interested in using it to identify inhibitors or activators of enzymes involved in tumorigenesis and related phenomena, for example, protein kinase C, ornithine decarboxylase, cyclic AMP-dependent protein kinase, the protein kinase domains of the insulin and EGF receptors, and the enzyme products of various cellular onc genes such as the <u>c-src</u> ($PP60^{src}$) or <u>c-ras</u> ($P20^{ras}$) genes.

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent serine/threonine protein kinase of fundamental importance in cellular growth control. PKC is activated endogenously by a wide variety of growth factors, hormones, and neurotransmitters, and has been shown to be a high affinity receptor for the phorbol ester tumor promoters as well as other agents which possess tumor promoting activity (for reviews see Nishizuka 1986; 1984; Ashendel, 1984). PKC has been shown to phosphorylate several intracellular protein substrates, including the epidermal growth factor (EGF) receptor (Hunter et al., 1984), pp60src (Gould et al., 1985), the insulin receptor (Bollag et al., 1986), p21 ras (Jeng et al., 1987), and many others (Nishizuka, 1986). Several laboratories have recently isolated cDNA clones encoding distinct forms of PKC, thus demonstrating that PKC is encoded by a multigene family (Ono et al., 1986, Knopf et al., 1986, Parker et al., 1986; Coussens et al., 1986; Makowske et al., 1986; Ohno et al., 1987; Housey et al., 1987). The multiple

forms of PKC exhibit considerable tissue specificity (Knopf, et. al., 1986; Brandt et al., 1987; Ohno, et al, 1987; Housey, et. al., 1987) which suggests that there may be subtle differences in the function(s) of each of the distinct forms. However, all of the cDNA clones which have been isolated thus far that encode distinct forms of PKC share at least 65% overall deduced homology at the amino acid level, and transient expression experiments with some of these cDNA clones have shown that they encode serine/threonine protein kinase activities which bind to, or are activated by, the phorbol ester tumor promoters (Knopf, et. al., 1986, Ono, et. al., 1987).

We used the PKCbeta1 cDNA clone for the present studies for the following reasons. With the exception of the brain, where its expression is very high, PKCbeta1 is expressed at very low levels in most tissues, and its expression is virtually undetectable in Rat 6 fibroblasts (see below). Thus, we reasoned that using this form would maximize the phenotypic differences observed between control cells and cells overexpressing the introduced form of PKC. The PKCbeta1 form is also of particular interest because within the PKC gene family its deduced carboxy terminal domain displays the highest overall homology to the catalytic subunit of the cyclic AMP-dependent protein kinase (PKAc) and the cyclic GMP-dependent protein kinase (PKG) (Housey et al., 1987). The latter observation suggests that PKAc, PKG, and the beta1 form of PKC may share a common ancestral serine/threonine protein kinase progenitor, and that the additional PKC genes may have been derived through evolutionary divergence from the beta1 form.

Agents which interact with certain structural proteins, such as actin and myosin, are also of interest. Mutations in the genes expressing these proteins may be involved in tumorigenesis and metastasization. Such interactions can lead to changes in cell phenotype which can be assayed by this method.

As is set forth in greater detail below, I have produced cell lines which overproduce protein kinase C (PKC). These cell lines, unlike the control cells, grow in soft agar even in the absence of the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). TPA has been shown to be a potent activator of PKC. When TPA is added to the growth medium the PKC-overproducing cell lines grow even better and form considerably larger colonies in soft agar. Furthermore, I have also tested known inhibitors of PKC activity which, as predicted, caused the PKC-overproducing cells to grow less well (or not at all) in soft agar. Thus, the direct utility of this method in identifying both activators or inhibitors of a gene product, in this case PKC, has been clearly demonstrated.

In additional studies with other genes, most notably the c-H-ras oncogene, the catalytic subunit of the cyclic AMP-dependent protein kinase, the c-myc oncogene, and certain cDNA clones encoding phorbol-ester inducible proteins, similar results have been obtained. Thus it is also clear that the method can be generalized to a wide variety of genes encoding proteins which are involved in cellular growth control in numerous cell types.

First I tested the capability of pMV7-based expression vectors (pMV7 is my preferred transfer vector) to produce several different types of proteins in various cell lines. I used the cDNA sequences encoding the following proteins: hypoxanthine/guanine phosphoribosyltransferase (HGPRT), the human T4 lymphocyte cell surface antigen, the human T8 lymphocyte cell surface antigen, and ornithine decarboxylase (ODC). In each case the pMV7 vector was capable of producing high levels of expression of the relevant gene thereby resulting in overproduction of the corresponding protein product.

Once I had verified that the pMV7-derived expression vector could reproducibly generate cell lines which stably overproduced proteins I then tested additional genes which encode proteins, other than PKC, which are also involved in cellular growth control.

In the first case I chose a cDNA clone which, in collaboration with others, I had previously isolated and characterized. This cDNA clone, designated TPA-S1, encodes a protein of as yet unknown function. Thus I chose this clone for the express purpose of testing the method under conditions where a gene is used which encodes a protein of unknown function. Since we had previously demonstrated that the transcription of the TPA-S1 gene is rapidly and strongly induced following the treatment of cells with tumor promoters such as TPA (Johnson, et al., 1987), it appeared that the TPA-S1 gene product played some role in cellular growth control, but we had no additional data regarding its function. Thus, to further test the Method, the TPA-S1 cDNA was cloned into the pMV7 expression vector, resulting in a plasmid construct designated

pMV7-TPA-S1, and cell lines were generated which stably overexpressed the TPA-S1 cDNA clone. These cell lines were generated exactly as described for the R6-PKCx series cell lines described below.

In this case the results were also qualitatively identical since the NIH/3T3-tpa-s1 cell lines which were generated that stably overproduced the tpa-s1 encoded protein were also found to exhibit anchorage independent growth in soft agar. This growth, furthermore, was dependent upon the level of TPA-S1 mRNA and TPA-S1 protein which was being synthesized in each cell line. Thus these cell lines could be used, in strictly analogous manner to the R6-PKCx series cell lines, for the development of either an inhibitor or an activator of the TPA-S1 protein using the Method as described herein. It should be noted, in addition, that in these experiments we were able to obtain cell lines overproducing TPA-S1 which exhibited plating efficiencies in soft agar in excess of 80%, even though the TPA-S1 gene does not appear to be an oncogene by the standard definition of the latter term.

In further experiments, I have tested the method using an activated c-H-ras oncogene (T24), again in analogous fashion to the techniques described herein, and again with analogous results to those described herein for both the PKC cDNA clone and the TPA-S1 cDNA clone. Thus, the Method can also be used for the rapid development of a p21 ras inhibitor. Taken together, the results described in this application demonstrate directly that the Method described herein is clearly generalizable to any gene which is involved in any way in cellular growth control.

The preferred protein inhibitor/activator drug screening method of the present invention comprises the following steps:

1. Construction of an expression vector which is capable of expressing the protein of interest in the selected host by inserting a gene encoding that protein into a transfer vector. The gene may be inserted 3' of a promoter already borne by the transfer vector, or a gegne and a promoter may be inserted sequentially or simultaneously.

2. Introduction of the expression vector (a) into cells which produce recombinant retrovirus particles, or (b) directly into host cells which will be used for subsequent drug screening tests (the resulting cells are called herein "test" cells).

In parallel, the transfer vector (i.e., the vector lacking the gene of interest and possibly a linked promoter but otherwise identical to the expression vector) is preferably also introduced into the host cells. Cell lines derived from this latter case will be used as negative controls in the subsequent drug screening tests. Alternatively, the unmodified host cells may be used as controls.

If 2a was employed, after an appropriate time (usually 48 hours), media containing recombinant virus particles is transferred onto host cells so as to obtain test or control cells.

3. The test and control cells are transferred to selective growth medium containing the appropriate drug which will only allow those cells harboring the

expression vector containing the selectable marker gene (as well as the gene or cDNA of experimental interest) to grow. After an appropriate selection time (usually 7-10 days), individual clones of cells (derivative cell lines) are isolated and propagated separately.

4. Each independent cell line is tested for the level of production of the POI. By this method, a range of cell lines is generated which overproduce from a few fold to more that 100-fold levels of the POI. In parallel, the control cell lines which contain only the transfer vector alone (with the selectable marker gene) are also assayed for their endogenous levels of the POI.

5. Each independent line is then tested for its growth capability in soft agar (or methocel, or any other similar matrix) of various percentages and containing different types of growth media until cell lines are identified which possess the desired growth characteristics as compared to the control cell lines.

6. Each cell line is also tested for its ability to form "foci", or areas of dense cellular growth, in tissue culture plates using media containing various percentages and types of serum (20%, 10%, 5% serum, fetal calf serum, calf serum, horse serum, etc.) and under various conditions of growth (e.g. addition of other hormones, growth factors, or other growth supplements to the medium, temperature and humidity variations, etc.). In these tests, the cells are maintained at post-confluence for extended periods of time (from two to eight weeks) with media changes every three days or as required. Such growth parameters are varied until cell lines are identified which posess the

desired foci formation capacity relative to the control cell lines under the identical conditions.

7. After a cell line possessing the required growth characteristics is identified, the cells are grown under the conditions determined in (5) above with the growth medium supplemented with either crude or purified substances which may contain biologically active agents specific to the POI. Thus, crude or purified substances possessing the latter properties can be rapidly identified by their ability to differentially alter the growth properties of the experimental cells (which overproduce the POI) relative to the control cells (which do not). This can be done rapidly even by simple observation with the naked eye, since the colonies which grow in soft agar after 2 weeks are easily seen even without staining, although they may be stained for easier detection.

Similarly, if the post-confluence foci formation assay is chosen, the foci which result after approximately two weeks can be easily seen with the naked eye, or these foci can also be stained. For screening very large numbers of compounds (tens of thousands or more), the entire procedure can be performed on 96 well tissue culture plates. This applies equally well for either the soft agar growth assay or the tissue culture foci formation assay. Results of the assays can be rapidly determined by measuring the relative absorbance of the test cells as compared to the control cells (at 500 nm, or another appropriate wavelength). Absorbance readings may be rapidly performed in a 96-well plate absorbance reader such as the "Titer-tek" plate reader, or any of several analogous apparatus currently available. In this

fashion, thousands of compounds could be screened per month for their biological activity with very low labor and materials costs.

Furthermorre, if antigen expression varies on teh test cells expressing high levels of the POI relative to the control cells, a simple Enzyme-Linked Immunoadsorption Assay (ELISA) could be performed and an antibody specific to the antigen.

While the assay may be performed with one control cell line and one test cell line, it is possible to use additional lines, tests lines with differing POI levels. Also additional sets of control/test lines, originating from other hosts, may be tested.

Specific examples implementing the series of steps described above are as follows.

Example 1

If one were interested in screening for a protein kinase C (PKC) inhibitor, cell lines would be generated and selected which grow well in soft agar (as a result of their overproduction of any form of PKC) and yet show an enhancement of their growth when compounds which are known to stimulate PKC are added to the growth medium. Appropriate control cells, of course, would not exhibit any of these characteristics. Screening for a potent PKC inhibitor could then be performed by searching for those substances which could selectively inhibit the soft agar (anchorage-independent) growth of the PKC-overproducing cell lines. Alternatively, since the PKC-overproducing cells also form small, dense foci in tissue culture,

one could also screen for substances which inhibit this foci formation.

Described below are the detailed aspects of the relevant techniques and methods used to apply the principles of the invention to the problem of developing a system useful for screening for potent inhibitors of protein kinase C (PKC), a high-affinity intracellular receptor for tumor-promoting agents. The cell lines which resulted from the application of this method are highly sensitive and responsive both to agents which activate PKC as well as to those which inhibit PKC.

Construction of plasmid pMV7

The construction of pMV7 was begun with plasmid pPyori which contains the polyoma virus origin of replication cloned into the unique BamHI site of pML-1 (Lusky and Botchan, 1981). This plasmid replicates in murine cells that contain the polyoma T antigen (Dailey and Basilico, 1985). Plasmid pMV (Perkins et al., 1983) was then cleaved with HincII and BglI. The 2.29 kb fragment that contains the Moloney Leukemia Virus Long Terminal Repeats (LTR), the packaging site, the splice donor site, and the proline tRNA binding site was isolated.

During the original construction of pMV (Perkins et al., 1983) 3.95 kb was removed from the MSV genome by cleaving with PstI. This left a PstI site situated 380 bps 3′ from the tRNA binding site and 308 bps from the 5′ end of the 3′ LTR (Reddy et al., 1981). Xho I linkers were added to the HincII-BglI fragment, and to pPyori after it had been cleaved with EcoRI and HindIII. The two fragments were ligated, after

activation of the linkers, and a plasmid, designated pMV-3, that contained the Moloney Virus control elements was isolated.

The unique EcoRI site was removed from this vector by digesting the plasmid with EcoRI and treating the linear molecules with T4 polymerase. These molecules were recircularized and a plasmid, pMV-4, lacking the EcoRI site, was isolated. An EcoRI linker was inserted into this plasmid at the PstI site between the Moloney LTRs; the resultant plasmid was designated pMV-5.

The dominant selectable marker (neo) was added to pMV-5. The first step was isolating a 1.9 kb BamHI-SalI fragment from pIPB1. This fragment contains the Herpes Simplex virus thymidine kinase (tk) promoter region and the coding sequence for the bacterial neo gene (neomycin phosphotransferase). This fragment was blunt-ended with T4 polymerase, ClaI linkers were added and the fragment was cloned into the Cla I site 165 bps 3′ to the EcoRI site in pMV-5, between the LTRs. This plasmid was designated pMV5-tk neo. The tk promoter has an EcoRI site 70 bps 5′ to the start of transcription. This EcoRI site was removed by partially digesting pMV5-tkneo with EcoRI, isolating the linear full length cut species, filling in the ends with T4 polymerase and recircularizing the molecule. A plasmid was chosen in which the EcoRI site previously present in the tk promoter sequence was removed, but the EcoRI site 537 bps 3′ to the start of transcription of the 5′ LTR was retained. This plasmid was designated pMV7 and a map of this plasmid is shown in Figure 1B.

This vector consists of the 5′ and 3′ Moloney Murine Leukemia Virus (MoMuLV) LTRs, the MoMuLV RNA packaging site 3′ to the 5′ LTR, an Eco RI cloning site, a modified Herpes Simplex Virus thymidine kinase (tk) promoter (lacking an Eco RI site at -79 bp), and the selectable marker gene neo. cDNA clones inserted into the EcoRI cloning site are under the transcriptional control of the 5′ LTR, whereas the neo gene is independently transcribed by the tk promoter. This structure favors maintenance of the functional integrity of the selectable marker without interfering with the expression of the 5′ (unselected) cDNA sequence. It is known (Maddon et al., 1986; Daley et al., 1987) that when various cDNA sequences are inserted into the EcoR1 site of pMV7 they can be readily transferred into recipient cells by virus-mediated passage, are stably expressed, and yield high-level production of the corresponding protein.

Nucleotide Sequencing and Expression Vector Construction

Nucleotide sequencing of the PKC cDNA clone RP58, a full-length clone isolated from a rat brain cDNA library, which corresponds to the previously reported clone RP41, was performed as previously described (Housey et al., 1987). The full-length cDNA sequence of RP58, which encodes PKCbeta1 (Figure 1A), was subcloned into the EcoRI site of plasmid pMV7 using standard methodology (Maniatis et al., 1983). The general structure of pMV7 is shown in Figure 1B. The construct resulting from insertion of the PKCbeta1 gene is designated pMV7-PKCbeta1.

Isolation of cell lines stably overexpressing PKC

20 ug of CsCl banded pMV7 or pMV7-PKCbeta1 plasmid DNA were transfected (Graham and van der Eb, 1973; as modified by Wigler et al., 1977) onto subconfluent "Psi-2" cells (Mann et al., 1985). After 48 hrs the culture medium was collected, filtered through a 0.45 um filter and stored at -70°C. Recipient subconfluent Rat-6 fibroblasts (5 x 10^5 per 10 cm plate) were infected with the virus-containing medium in 2 ug/ml polybrene for 48 hrs. The cells, grown to confluence, were then trypsinized and replated in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (CS) (Flow Laboratories) with 200 ug/ml of the neomycin derivative G418 (Geneticin). Resistant colonies were cloned by ring isolation after 1 week of G418 selection.

After approximately ten days of growth in selective medium, ten individual G418-resistant clones were isolated and maintained independently in G418-containing medium. These lines were designated R6-PKCbeta11 through R6-PKCbeta110 (abbreviated as R6-PKC1 through R6-PKC10). In parallel, a set of control Rat 6 lines was generated by transfection of the plasmid pMV7 (lacking the PKC cDNA insert) onto W-2 cells, infection of recipient Rat-6 cells, and selection for G418 resistance as described above for plasmid pMV7-PKCbeta1. Similarly, after ten days of growth in the G418-containing medium, five individual, well-isolated G418-resistant clones were then isolated and maintained independently. These control lines were designated R6-C1 through R6-C5.

RNA Isolation and Blot Hybridization Analyses

Poly A+ RNA isolations, gel electrophoresis, and blot hybridization analyses were performed as previously described (Housey, et. al., 1985). RNA molecular weight markers were obtained from Bethesda Research Laboratories. The 2.7kb cDNA insert of RP58 (see above) was subcloned into plasmid pKS(+) (Stratagene Cloning Systems) to yield a plasmid designated pS2-RP58. A ^{32}P-labelled probe was prepared from pS2-RP58 and used under high-stringency hybridization conditions as previously described (Housey et al., 1987).

Purification and Assay of PKC Activity From Tissue Culture Cells

The total PKC activity (membrane-associated plus cytosolic) present in cultured cells was determined after partial purification of cellular extracts as follows.

Three 10 cm plates of confluent cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) and then 10 ml of homogenization buffer (20 mM Tris, pH 7.5, 5 mM EGTA, 5mM EDTA, 15 mM 2-mercaptoethanol, 10 ug/ml soybean trypsin inhibitor, 10 ug/ml leupeptin, 40 ug/ml phenylmethylsulfonyl fluoride), containing 0.1% Triton X-100 were added. The cells were then scraped from each of the plates, pooled and disrupted with 25 strokes in a Dounce homogenizer. The homogenate was transferred to a 15 ml disposable polystyrene tube, centrifuged at 2000 x g for 5 minutes at 4°C, and the supernatant was loaded onto a 1 ml DEAE Sephacel column previously equilibrated with 10 ml homogenization buffer, at 4°C. The column was washed with 10 ml homogenization buffer

and then the bound enzyme was eluted with 3 ml of homogenization buffer containing 0.5 M NaCl. Total protein concentrations were determined by the method of Bradford (1976).

The PKC activity present in the above-described partially purified cell extracts above was assayed immediately after isolation. The synthetic peptide R-K-R-T-L-R-R-L, corresponding to amino acids 651-658 of the epidermal growth factor receptor (Ullrich et al., 1984), was synthesized on an Applied Biosystems model 430A peptide synthesizer, purified by high-performance liquid chromatography, lyophilized, and stored at -20 oC. The Threonine at position 654 is an in vivo substrate for PKC (Hunter, et al., 1984; Davis and Czech, 1985). This synthetic peptide is a highly specific substrate for PKC activity _in vitro_. (Watson et al., 1987; Woodgett, et al., 1986).

The purified material was then redissolved in sterile water at a final concentration of 100 uM and used as the phosphoacceptor substrate in the PKC assays. The general method of assay has been published in detail elsewhere (O'Brian et al., 1985). In most cases, 100 uM synthetic peptide was substituted for 2 mg/ml histone III-S as the phosphoacceptor substrate.

Cell extracts were prepared 48 hours after the cells had reached confluence. As shown in Table 1(a), eight of the ten cell lines generated by infection with the pMV7-PKCbeta1 construct (lines R6-PKC1, R6-PKC3, and R6-PKC5 through R6-PKC10) contained marked increases in PKC activity when compared to the control lines (R6-C1, -C2, and -C3). It is remarkable that cell line R6-PKC3 contained a 53 fold higher level of

PKC activity than that present in the control cells. Two of the lines (R6-PKC2 and R6-PKC4), however, did not display a significant increase in PKC activity, yet they presumably had integrated the pMV7-PKC construct as evidenced by their continued G418 resistance. Subsequent studies verified that these two cell lines contained deletions in the cDNA clone encoding PKCbeta1.

In additional studies I found that the very high PKC activity in extracts of R6-PKC3 seen in the presence of 1mM Ca^{2+} and phosphatidylserine was also apparent in the presence of 1mM EGTA, 100 ng/ml TPA and phosphatidylserine. This very high activity was also seen when we employed histone III-S, rather than the above-described synthetic peptide, as the substrate for phosphorylation. Furthermore, even after 24 weeks of continous growth and serial passage, the cell lines R6-PKC1 through R6-PKC6 displayed essentially the same levels of PKC activity shown in Table 1.

Autophosphorylation and Gel Electrophoresis of PKC

Cell extracts purified as described above were incubated under conditions which favor the autophosphorylation of PKC, as follows. One hundred ug of partially purified protein extract was incubated in a reaction mixture containing 80 ug/ml phosphatidylserine, 1 mM CaCl2 (or 1 mM EGTA and 100 ng/ml TPA), 5 mM MgCl2, and 30 uM ATP containing 100 uCi [gamma-^{32}P]ATP (New England Nuclear, NEG035). The purity of the radioactive ATP is critical to obtain reproducible autophosphorylation of PKC.

Under these conditions, it has been previously shown that PKC undergoes an autophosphorylation reaction which results in the phosphorylation of several sites on the intact enzyme (Walton et al., 1987; Huang et al., 1986, Woodgett and Hunter, 1986; Kikkawa, 1982).

Reactions were incubated at room temperature for 10 minutes and then stopped by the addition of SDS-PAGE loading buffer containing 2-mercaptoethanol. This material was then subjected to discontinuous SDS-PAGE by a modification of the method of Laemmli (1970). Twenty ug of total protein were loaded onto each lane. Following electrophoresis, the gels were fixed in 50% acetic acid, 10% ethanol, dried, and autoradiographed on Kodak XAR-5 film.

Autoradiographs of these gels (Figure 2) revealed that the extracts prepared from four cell lines that had high PKC activity (R6-PKC1, -PKC3, -PKC5, and -PKC6, see Table 1(a)) displayed a prominent phosphorylated protein band which was about 75 kd in size, corresponding to the size of an autophosphorylated preparation of PKC obtained from rat brain (Huang et al., 1986; Housey et al., 1987). When examined in an immunoblot assay this 75 kd band also reacted with an antibody to the beta1 form of PKC (Jaken and Kiley, 1987). The control cell lines R6-C1, -C2 and -C3, and the cell lines R6-PKC2 and R6-PKC4, which did not have increased levels of PKC (see Table 1(a)) did not show this 75 kd phosphorylated band (Figure 2), nor did they contain any bands which reacted with the antibody to the beta1 form of PKC. It is of interest that the samples obtained from the four cell lines that produced high levels of PKC also

displayed weaker but distinct phosphorylated protein bands that were about 73, 60, and 49 kd in size, which were not seen (or only faintly detected) in the extracts from cells that did not have increased levels of PKC (Figure 2). These bands may represent degradation fragments of the 75 kd PKC molecules, or specific cellular proteins that are phosphorylated by PKC.

The above-described phosphorylated protein bands were seen when either 1 mM Ca2+ plus phosphatidylserine or 100 ng/ml TPA plus phosphatidylserine were used as cofactors for PKC activation (compare even and odd numbered lanes in Figure 2). When, however, extracts from cell lines producing high levels of PKC were incubated in an autophosphorylation reaction in the absence of such cofactors, the 75 kd band and the additional smaller bands described above were not detected. These results, taken together with the negative results obtained with extracts from the control cells (Figure 2), clearly indicate that the phosphorylated bands reflect PKC activity.

Phorbol Ester (^{3}H-PDBU) Binding Assays

Since it has been shown that PKC is a high affinity intracellular receptor for the phorbol ester tumor promoters (for review, see Nishizuka, 1986), I also assayed a subset of the cell lines for 3H-PDBu binding using a previously described intact cell assay (Horowitz et al., 1981).

Cells were plated at 1 x 10^5 per 4 cm well on day 1, the medium was changed on day 2 and the cells then assayed on day 3. The monolayer was washed with 4 ml of

DMEM (without serum), then 2 ml of DMEM containing 50 nM ^{3}H-PDBU (New England Nuclear; 8.3 Ci/mmol) was added and the plates were incubated for 1 hr at 37oC to determine total binding. The fraction of the total binding that represented specific binding was determined by the addition of a 1000-fold excess of unlabelled PDBU (LC Services) to the 3H-PDBU stock (Horowitz et al., 1981). The plates were washed 3x with 4 ml ice-cold PBS. The cells were solubilized in 1 ml 1% SDS/10 mM DTT for 2 hrs at 37^{o}C. The lysate was transferred to a scintillation vial and counted. Replica plates were used to determine the number of cells per plate and the specific binding data expressed as nanomoles 3H-PDBu bound/10^{6} cells. Scatchard analyses were performed as previously described (Horowitz et al., 1981).

I found that the R6-PKC cell lines 1,3,5 and 6, all of which had high PKC enzyme activity, also had a marked increase in 3H-PDBu binding, whereas the cell line R6-PKC4, which did not display a significant increase in PKC activity, did not show an increase in 3H-PDBu binding when compared to the two control cell lines R6-C1 and R6-C2 (Table 1(a)). Scatchard analyses of the control cell line R6-C1 and of the R6-PKC3 cell line, performed as previously described (Horowitz et al., 1981), indicated that the number of high affinity receptors in the two cell lines was 1.6 X 10^{5} and 1.4 X 10^{6}, respectively. The affinity constants were approximately the same in both cell lines (Kd = 16 nM). Thus, under the assay conditions used, the R6-PKC3 cells contain about ten times the level of high affinity phorbol-ester binding sites as the control cells. It is apparent that the cell lines that express

very high levels of PKC also have a significant increase in phorbol ester binding sites.

Assays for PKC-related RNA transcripts

In view of the above results, it was of interest to analyze the poly A+ RNA fraction of several of the cell lines described in Table 1(a) for the size and abundance of RNA transcripts containing sequences homologous to PKCbeta1.

Poly A+ RNA was separated on 1% agarose, 6%-formaldehyde gels, blotted onto nylon membranes, hybridized to a 32P-labelled DNA probe prepared from the full-length PKCbeta1 cDNA, and autoradiographed, as previously described (Housey, et al., 1986). As shown in Figure 3, the lines that contained elevated levels of PKC activity (R6-PKC1,-PKC3,-PKC5, and -PKC6, see Table 1(a)) contained a prominent 6.6 kb RNA species which corresponds to the predicted size for a mRNA transcript that initiates in the 5′ LTR and terminates in the 3′ LTR of the pMV7-PKCbeta1 construct. This transcript was most abundant in the R6-PKC3 cell line (Figure 3) which also expresses the highest level of PKC activity (Table 1).

On the other hand, lines R6-PKC2 and R6-PKC4, which showed no significant elevation of PKC activity (Table 1(a)), produced truncated mRNA's of approximately 5 kb and 4.8 kb, respectively. The abundance of the latter transcripts was much lower than that of the 6.6 kb transcripts present in the cell lines that expressed high levels of PKC. The neo+ phenotype and the lack of PKC activity in cell lines R6-PKC2 and R6-PKC4 suggest that the truncated mRNAs reflect deletions in PKC coding sequences in the

integrated constructs carried by these cell lines. Indeed, genomic DNA blot hybridizations of clones R6-PKC2 and R6-PKC4 indicated that they contained deletions in the PKCbeta1 cDNA sequence. Neither in the parental Rat 6 cell line, nor in any of the experimental cell lines derived from these cells, was it possible to detect evidence of an endogenous transcript homologous to the PKCbeta1 probe (Figure 3). Thus, in these cells, there is negligible expression of the endogenous gene encoding PKCbeta1.

Assays of Growth in Monolayer Culture and in Soft Agar

Cells were seeded at a density of 10^4/plate in a series of 60 mm plates, in 5 ml DMEM plus 10% CS. Twenty-four hours later, cells in triplicate plates were trypsinized and counted. This point was designated "day 0." The remaining cultures were then grown in the respective medium (i.e. plus or minus 100 ng/ml TPA) with fresh medium changes twice per week. Cell counts per plate were then determined on triplicate plates during the remainder of the growth curve (Figure 3). The results obtained were analyzed for exponential doubling time and saturation density (Table 2). To assess growth in soft agar (anchorage independence), 2 X 10^4 cells were suspended in 2 ml of 0.3% Bacto agar (Difco Laboratories, Detroit, MI) in DMEM containing 20% fetal calf serum (FCS) and overlayed above a layer of 5 ml of 0.5% agar in the same medium, on 60 mm petri dishes. The cells were then overlaid with DMEM plus 20% FCS every 4 days. At the end of 30 days, colonies were stained with the vital stain 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolinium chloride hydrate (INT) (Sigma Chemical Co., St. Louis, MO) for 48 hours at 37 oC, in an incubator with

5% CO_2 (Schaeffer and Friend, 1976), and the number of colonies counted under low power on an inverted phase microscope. The data are expressed as "cloning efficiency", i.e. number of colonies greater than 0.05 mm per plate X 100 divided by the number of cells originally seeded per plate.

Screening of a Known Activator of PKC

We found that cell lines which overproduce PKC display an exaggerated morphologic response to 12-O-tetradecanoyl phorbol-13-acetate (TPA) and altered growth control.

Changes in Morphology

To further characterize the phenotypic changes which occurred in the cell lines that overproduce PKCbeta1, lines R6-PKC3 and R6-PKC5, which contain 53- and 20-fold elevations of PKC activity, respectively, were first examined in detail with respect to their morphology, in comparison to the control cell line R6-C1. As shown in Figure 4, Panel A, in the absence of TPA treatment all three cell lines showed the characteristic fusiform morphology of monolayer cultures of the normal parental Rat 6 fibroblast cell line. At 24 hours after treatment with 100 ng/ml TPA (Figure 4, R6-C1: panel D), the control cell line displayed more elongated and dendritic cells and a criss-cross pattern, changes previously seen shortly after rodent fibroblasts are treated with TPA (Boreiko et al., 1980). These changes were, however, much more dramatic when the R6-PKC3 and R6-PKC5 cells were treated with TPA (R6-PKC3 and R6-PKC5, Panel D). This was particularly striking with the R6-PKC3 cells (which

express the highest level of PKC) since they displayed very long cytoplasmic processes and numerous refractile cell bodies.

By 48 hours following exposure to TPA the morphology of the control cell line R6-C1 had returned to its normal appearance (Figure 4, Panel E). On the other hand, the R6-PKC3 cells, and to a lesser extent the R6-PKC5 cells, continued to display an altered morphology. All of the cell lines were then exposed to a second, fresh dose of TPA (100 ng/ml) and examined 24 hours later (Figure 4, Panel F), i.e., 72 hours after the first dose of TPA. The control cells failed to respond, in terms of morphologic change, to the second dose of TPA whereas the R6-PKC3 cells continued to display their altered morphology as well as an increase in cell density. The R6-PKC5 cells displayed only slight changes in morphology in response to the second dose of TPA.

Presumably, the very high level of constitutive production of PKC in the R6-PKC3 cell line is responsible for their exaggerated morphologic response to TPA as well as the failure of these cells to display the usual refractory response to TPA following an initial exposure. In normal cells, the latter response appears to be due to "down-regulation" of endogenous PKC activity. Since the R6-PKC5 cells have an intermediate level of PKC, it is not surprising that their morphologic responses to TPA are intermediate between those of the control cells and the R6-PKC3 cells.

Growth Curves

It was also of interest to determine the growth rates of these cells in monolayer culture. Detailed growth curves were performed on R6-C1, R6-PKC3 and R6-PKC5 cells in 10% calf serum and DMEM medium, in the absence and presence of 100 ng/ml TPA. The data obtained are summarized quantitatively in Table 2.

In the absence of TPA, the R6-C1 control cell line displayed the longest doubling time (26.4 hours) and the lowest saturation density (3.4 x 10^6 cells/plate); the R6-5 cell line had a shorter doubling time (24.9 hours) and a higher saturation density (4.8 x 10^6 cells/plate); and the R6-PKC3 cell line had the shortest doubling time (24.2 hours) and the highest saturation density (5.7 x 10^6 cells/plate). The presence of TPA decreased the doubling times, and also increased the saturation densities of all three cell lines, but the enhancement by TPA was particularly striking in the case of R6-PKC3 (Table 2).

When the cell lines were maintained in the presence of TPA for a longer period of time, the R6-PKC3 cells, but not the R6PKC5 or R6-C1 cells, showed a decline in cell density. The latter effect was due to the fact that when the R6-PKC3 cells were maintained at high cell density in the presence of TPA they became less adhesive and tended to detach from the plate.

Thus, even in the absence of TPA the R6-PKC3 cells, (which have the highest level of PKC), and to a lesser extent the R6-PKC5 cells (which have an intermediate level of PKC), exhibit an enhancement of their growth properties which is even greater than that seen when the R6-C1 control cells are grown in the

presence of TPA. Moreover, in the presence of TPA these differences in growth properties between the control and R6-PKC3 cells are even more striking.

Foci Formation

In additional studies, monolayer cultures were maintained at post-confluence for an extended period of time (28 days), with media changes every 3 days, in the absence of TPA. Whereas the control R6-C2 cell line remained a fairly uniform monolayer, after about 21 days the R6-PKC3 cell line developed numerous dense foci which were approximately 0.1-0.3 mm in diameter (Figure 5). Furthermore, the R6-PKC3 cultures displayed numerous cells with a highly vacuolated cytoplasm which were scattered throughout the monolayer, but were not seen in the R6-C2 control culture. When the dense foci seen in the R6-PKC3 culture were picked and further passaged they grew like the parental R6-PKC3 cells and did not display a morphology typical of malignantly transformed cells. It may be surmised that these dense foci, and the vacuolated cells, reflect physiologic rather than genetic changes induced by the high level of PKC activity.

Growth on Soft Agar

I also assayed these cell lines for their ability to form colonies in soft agar, since with rodent cells the acquisition of anchorage-independent growth often correlates with tumorigenicity (Freedman and Shin, 1974). As shown in Figure 6, when 2×10^4 cells were plated in 0.3% soft agar, both the R6-PKC3 and the R6-PKC5 cells formed numerous small colonies, whereas

the control R6-C1 cells (and the parental Rat 6 cell line) failed to grow and persisted as single cells. In addition, when TPA (100 ng/ml) was added to the agar medium, the colony sizes and cloning efficiencies of the R6-PKC3 and RC-PKC5 cells were enhanced (Figure 6 and Table 2), but the R6-C1 cells still failed to grow in agar.

The cloning efficiencies and colony sizes of the R6-PKC3 cultures were always greater than those of the R6-PKC5 cultures, both in the absence and presence of TPA (Table 2), presumably reflecting the higher level of PKC activity in the former cell line. Thus, it is clear that the overproduction of PKC is associated with the acquisition of anchorage-independent growth in Rat 6 cells. The sizes of the colonies formed in agar by the PKC cell lines are smaller than those formed by Rat 6 cells transformed by an activated c-H-ras oncogene, which have a diameter of about 0.5 - 1.0 mm.

Further Screening of Activators and Inhibitors of PKC

Additional compounds tested included the tumor promoters teleocidin, aplysiatoxin, and mezerein, all of which are known activators of PKC (O'Brian, et al., CSH, 1985). Furthermore, a known inhibitor of PKC, H-7 (Kawamoto and Hidaka, 1984), also modulated the growth of the cells in the expected manner.

Moreover, this method has been used to establish that the anti-estrogen tamoxifen (O'Brian, et al, Cancer Res., 1985), which inhibits PKC enzyme activity in a cell-free assay, is capable of completely inhibiting the growth in agar of all of the cell lines overproducing PKC. Inhibition of the growth of the

R6-PKC3 cells in agar in the presence of tamoxifen provided critical evidence that tamoxifen could inhibit PKC-mediated stimulation of cellular growth.

Furthermore, the concentration of the inhibitor necessary to completely inhibit the growth of each cell line was roughly proportional to the amount of PKC being overproduced in that particular cell ine. In other words, there is a direct relationship between the molar amount of inhibitor required to prevent cell growth and the molar amount of PKC present in each cell line.

In addition, I have demonstrated the analogous relationship between the molar amounts of PKC activators and the molar amounts of PKC present in cells. In other words, there is a direct relationship between the molar amount of activator required to stimulate cell growth and the molar amount of PKC present in cells. Thus, this work establishes, for the first time, the fact that stable overproduction of a protein in mammalian cells can result in a novel cellular phenotype(s) (in this case anchorage independence) which can be directly modulated by chemical agents which interact with the protein.

Example 2

If one were interested in screening for a potent inhibitor of the c-H-ras oncogene product (the p21 protein) then one would generate cells which grow well in soft agar with appropriate media conditions when p21 is stably overproduced at a certain level, but not at all when p21 is present at wild type levels. Screening for a potent p21 inhibitor could then be performed as described in Example 1 above.

Example 3

The same basic techniques would also apply to genes (or cDNA sequences) which have been mutated either by laboratory design (e.g. site-directed mutagenesis) or as a result of naturally occurring events. Thus, any of the known point mutations in the ras oncogene which result in greater capability by the mutated gene to transform normal cells to cancerous ones could be employed in the same basic procedures as described above

Further Modifications

Use of any expression vector capable of stably overexpressing a given gene in a recipient cell could be used with success in the procedures described herein. The retroviral vector which I used here was particularly well suited to the problem since I had designed it specifically for these purposes. However, other similar vector systems would work. Also, one could do co-transfection of an experimental gene inserted in one plasmid vector along with a second plasmid containing the selectable marker gene (rather than having both the experimental gene and the selectable marker gene on the same plasmid vector). This is more difficult and less efficient than using the pMV7 vector, but it would work to some extent.

Any growth medium, in addition to soft agar or methocel, which tends to prohibit the growth of normal, non-transformed cells, could also be used.

A culture of E. coli DH1 bearing the plasmid denoted pMV7-RP58 (pMV7-PKC beta1), was deposited under

the Budapest Treaty with the American Type Culture Collection on February 11, 1988, ATCC No. 67654. The deposit of this plasmid is not to be construed as an admission that the deposit is required for enablement or that the disclosure is limited to the deposited vector or gene.

TABLE 1(a)

PKC Activity and Phorbol Ester Binding in Rat 6 Cells Infected with pMV7 or pMV7-PKCbeta1 Constructs

Cell Line	PKC Activity Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control	3H-PDBU Binding (pmol/10^6 cells)
R6-C1	100	____	1.6
R6-C2	85	____	1.3
R6-C3	150	____	1.5
mean + s.d.	100 + 34	1	
R6-PKC1	2480	23	12.7
R6-PKC2	85	1	ND
R6-PKC3	5840	53	9.9
R6-PKC4	190	2	0.7
R6-PKC5	2200	20	5.8
R6-PKC6	4600	42	7.1
R6-PKC7	2150	20	ND
R6-PKC8	3280	30	ND
R6-PKC9	4990	45	ND
R6-PKC10	5050	46	ND

TABLE 1(b)

PKC Activity in NIH-3T3 Cell Lines
Infected with pMV7 or pMV7-PKCbeta1 Constructs

NIH-3T3 Cell lines (Controls)	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
3T3-C1	110	-----
3T3-C2	150	-----
3T3-C3	90	-----
mean +/- s.d. (control lines)	115 +/- 30	
NIH-3T3 PKC-Overproducing Cell Lines		
3T3-PKC1	2570	22
3T3-PKC2	3640	32
3T3-PKC3	1960	17
3T3-PKC4	1240	11
3T3-PKC5	4190	36
3T3-PKC6	2110	18
3T3-PKC7	5050	44

Table 1(c)

PKC Activity in C3H-10T1/2 Cell Lines Infected with pMV7 or pMV7-PKCbeta1 Constructs

C3H-10T1/2 Cell lines (Controls)	PKC Specific Activity (pmol/min/mg prot)	Fold Increase Relative to Control
C3H-C1	115	-----
C3H-C2	155	-----
C3H-C3	130	-----
C3H-C4	185	-----
mean +/- s.d.	145 +/- 30	
C3H-10T1/2 PKC-Overproducing Cell Lines		
C3H-PKC1	2340	16
C3H-PKC2	5010	35
C3H-PKC3	950	7
C3H-PKC4	1360	9
C3H-PKC5	4340	30
C3H-PKC6	7460	51

Legend to Table 1

Control cell lines were obtained by infecting rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells with the pMV7 vector itself (lacking the PKC cDNA insert) whereas the PKC-overproducing cell lines were obtained from rat-6-fibroblast, NIH-3T3 and C3H-10T1/2 cells infected with the pMV7-PKCbeta construct, as described above. Total PKC activity was partially purified from each of the cell lines and assayed in the presence of 1 mM Ca2+ and 80 ug/ml phosphatidylserine, using the synthetic peptide R-K-R-T-L-R-R-L as substrate. Specific activity is reported as the amount of incorporation of 32P into the synthetic peptide substrate per milligram of protein per minute. All assays were done in duplicate and varied by less than 10%.

TABLE 2

GROWTH PROPERTIES OF RAT 6 CELL LINES OVERPRODUCING PKC AND THEIR RESPONSES TO TPA TREATMENT

		Growth in Culture			
		Monolayer		Agar	
Cell Line	TPA Add	Doubling Time (hrs)	Saturat. Density ($x10^6$)	Effic. (%)	Colony Size (mm)
R6C1	-	26.4	3.4	0	---
(control)	+	24.6	4.2	0	---
R6-PKC3	-	24.2	5.7	25.1	0.10 - 0.15
(test)	+	21.5	10.0	29.7	0.15 - 0.35
R6-PKC5	-	24.9	4.8	17.3	0.05 - 0.10
(test)	+	22.9	7.0	34.7	0.10 - 0.15

Table 2. The cells were grown as described above. The "doubling times" relate to the initial exponential phase of cell growth and the "saturation density" represents the number of cells per 6 cm plate on day 11. The data are taken from the experiment shown in Figure 3.

Table 3

Inhibition of Growth in Agar of PKC-Overproducing Cell Lines Using Various Inhibitors

Inhibitor	Cell Line	Conc. Inhibitor (uM)	Effic. of Growth In Agar (%)
H-7	R6-PKC3	0	27
H-7	R6-PKC3	2	25
H-7	R6-PKC3	5	22
H-7	R6-PKC3	10	17
H-7	R6-PKC3	50	4
H-7	R6-PKC3	100	0
H-7	R6-PKC5	0	20
H-7	R6-PKC5	2	21
H-7	R6-PKC5	5	16
H-7	R6-PKC5	10	4
H-7	R6-PKC5	50	1
H-7	R6-PKC5	100	0
Tamoxifen	R6-PKC3	0	25
Tamoxifen	R6-PKC3	5	27
Tamoxifen	R6-PKC3	20	22
Tamoxifen	R6-PKC3	50	15
Tamoxifen	R6-PKC3	100	6
Tamoxifen	R6-PKC3	200	0
Tamoxifen	R6-PKC5	0	19
Tamoxifen	R6-PKC5	5	19
Tamoxifen	R6-PKC5	20	17
Tamoxifen	R6-PKC5	50	8
Tamoxifen	R6-PKC5	100	0
Tamoxifen	R6-PKC5	200	0
Staurosporine	R6-PKC3	0	29
Staurosporine	R6-PKC3	0.001	29
Staurosporine	R6-PKC3	0.005	26
Staurosporine	R6-PKC3	0.010	15
Staurosporine	R6-PKC3	0.050	7
Staurosporine	R6-PKC3	0.250	0
Staurosporine	R6-PKC5	0	22
Staurosporine	R6-PKC5	0.001	21
Staurosporine	R6-PKC5	0.005	17
Staurosporine	R6-PKC5	0.010	10
Staurosporine	R6-PKC5	0.050	2
Staurosporine	R6-PKC5	0.250	0

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CLAIMS

1. A method of determining whether a substance is an inhibitor or activator of a protein whose production by a cell evokes a responsive change in a phenotypic characteristic other than the level of said protein in said cell per se, which comprises:

(a) providing a first cell line which produces said protein and exhibits said phenotypic response to the protein;

(b) providing a second cell line which produces the protein at a lower level than the first cell line, or does not produces the protein at all, and which exhibits said phenotypic response to the protein to a lesser degree or not at all;

(c) incubating the substance with the first and second cell lines; and

(d) comparing the phenotypic response of the first cell line to the substance with the phenotypic response of the second cell line to the substance.

2. The method of claim 1 wherein the response is one observable with the naked eye.

3. The method of claim 1 wherein the response is a change in a cultural or morphological characteristic of the cell.

4. The method of claim 1 wherein the response is a change in the ability of the cell line to grow in an anchorage-independent fashion.

5. The method of claim 1 wherein the response is a change in the ability of the cell line to grow on soft agar.

6. The method of claim 1 wherein the response is a change in foci formation in cell culture.

7. The method of claim 1 wherein the response is a change in the ability of the cells to take up a selected stain.

8. The method of claim 1 in which the protein is an enzyme.

9. The method of claim 8 wherein increased activity of the enzyme is correlated with increased tumorigenesis.

10. The method of claim 9 in which the enzyme is a protein kinase C enzyme or a fragment, domain or subunit of a receptor which has protein kinase C activity.

11. The method of claim 9 wherein the enzyme is ornithine decarboxylase.

12. The method of claim 9 in which the protein is the expression product of an oncogene.

13. The method of claim 1 in which the substance

is a suspected inhibitor of the biological activity of the protein.

14. The method of claim 1 in which the substance is a suspected activator of the biological activity of the protein.

15. The method of claim 1, wherein said first cell line is obtained by introducing a gene encoding the protein of interest into a host cell, said gene being under the control of a promoter functional in the host cell, whereby said gene is expressed.

16. The method of claim 15, wherein the gene is introduced into the host cell by means of a first genetic vector into which the gene has been inserted, and said second cell line is obtained by introducing into a similar host cell a second genetic vector essentially identical to the first genetic vector except that it does not bear said gene insert.

17. The method of claim 15 wherein the gene is introduced into the host cell by means of a retroviral vector.

18. The method of claim 15 in which the host cell line essentially does not produce the protein.

19. The method of claim 15 in which the host cell line is a rat-6 fibroblast cell line.

20. The method of claim 3 in which the response is a change in the differentiation state of the cell.

21. A test kit for determining whether a substance is an inhibitor or activator of a protein whose production evokes a responsive change in a phenotypic characteristic other than level of said protein in said cell per se, which comprises:

(a) a first cell line which produces the protein and exhibits said phenotypic response thereto; and

(b) a second cell line which produces the protein at a lower level than the first cell line, or does not produce the protein, and which exhibits said phenotypic response to the protein to a lesser degree or not at all.

22. The test kit of claim 21, wherein the level of production of the protein in the first cell line is at least five times the level of production of the protein in the second line.

23. The test kit of claim 21, wherein the phenotypic response to expression of the protein is selected from the group consisting of changes in growth rate, saturation density, plating efficiency in soft agar, colony size in soft agar, and combinations thereof.

24. The method of claim 1 wherein the response is a change in an antigenic characteristic of the cell.

FIG. 1.

```
             10          20          30          40          50          60
              *           *           *           *           *           *
GAA TTC CGC CTC TCC GGG CTT ACA GCC CGC GGT CCC GCC GCC CCG GGG CCG CCA CCT CTC
             70          80          90         100         110         120
              *           *           *           *           *           *
GGG GCT CCC CCC AGT CCC CGC GCG CGC AAG ATG GCT GAC CCG GCT GCG GGG CCG CCG CCG
                                        Met Ala Asp Pro Ala Ala Gly Pro Pro Pro
            130         140         150         160         170         180
              *           *           *           *           *           *
AGC GAG GGC GAG GAG AGC ACG GTG CGC TTC GCC CGC AAA GGG CCC CTC CGG CAG AAG AAC
Ser Glu Gly Glu Glu Ser Thr Val Arg Phe Ala Arg Lys Gly Pro Leu Arg Gln Lys Asn
            190         200         210         220         230         240
              *           *           *           *           *           *
GTG CAC GAG GTG AAG AAC CAC AAA TTC ACC GCC CGC TTC TTC AAG CAG CCC ACC TTC TGC
Val His Glu Val Lys Asn His Lys Phe Thr Ala Arg Phe Phe Lys Gln Pro Thr Phe Cys
            250         260         270         280         290         300
              *           *           *           *           *           *
AGC CAC TGC ACC GAC TTC ATT TGG GGC TTC GGG AAG CAG GGA TTC CAG TGT CAA GTC TGC
Ser His Cys Thr Asp Phe Ile Trp Gly Phe Gly Lys Gln Gly Phe Gln Cys Gln Val Cys
            310         320         330         340         350         360
              *           *           *           *           *           *
TGC TTT GTT GTA CAC AAG CGC TGC CAT GAA TTC GTC ACG TTC TCC TGC CCT GGT GCA GAC
Cys Phe Val Val His Lys Arg Cys His Glu Phe Val Thr Phe Ser Cys Pro Gly Ala Asp
            370         380         390         400         410         420
              *           *           *           *           *           *
AAG GGC CCG GCC TCT GAT GAC CCA CGG AGC AAA CAC AAG TTT AAG ATC CAC ACC TAC TCC
Lys Gly Pro Ala Ser Asp Asp Pro Arg Ser Lys His Lys Phe Lys Ile His Thr Tyr Ser
            430         440         450         460         470         480
              *           *           *           *           *           *
AGC CCT ACC TTC TGT GAC CAC TGT GGA TCA CTG CTG TAT GGG CTC ATC CAC CAG GGG ATG
Ser Pro Thr Phe Cys Asp His Cys Gly Ser Leu Leu Tyr Gly Leu Ile His Gln Gly Met
            490         500         510         520         530         540
              *           *           *           *           *           *
AAA TGC GAC ACC TGT ATG ATG AAT GTC CAC AAG CGC TGC GTG ATG AAC GTC CCC AGC CTC
Lys Cys Asp Thr Cys Met Met Asn Val His Lys Arg Cys Val Met Asn Val Pro Ser Leu
            550         560         570         580         590         600
              *           *           *           *           *           *
TGT GGC ACC GAC CAC ACA GAA CGC CGT GGC CGC ATC TAC ATC CAG GCC CAC ATC GAC AGG
Cys Gly Thr Asp His Thr Glu Arg Arg Gly Arg Ile Tyr Ile Gln Ala His Ile Asp Arg
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FIG. 1. CONT.

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          610       620       630       640       650       660
            *         *         *         *         *         *
GAG GTC CTC ATC GTT GTT GTA AGA GAT GCT AAA AAT CTG GTA CCT ATG GAC CCC AAC GGC
Glu Val Leu Ile Val Val Val Arg Asp Ala Lys Asn Leu Val Pro Met Asp Pro Asn Gly
          670       680       690       700       710       720
            *         *         *         *         *         *
TTG TCA GAT CCC TAC GTA AAA CTG AAA CTG ATC CCT GAT CCC AAA AGT GAG AGC AAG CAG
Leu Ser Asp Pro Tyr Val Lys Leu Lys Leu Ile Pro Asp Pro Lys Ser Glu Ser Lys Gln
          730       740       750       760       770       780
            *         *         *         *         *         *
AAG ACC AAG ACT ATC AAA TGC TCC CTC AAC CCG GAG TGG AAC GAA ACC TTC AGA TTT CAG
Lys Thr Lys Thr Ile Lys Cys Ser Leu Asn Pro Glu Trp Asn Glu Thr Phe Arg Phe Gln
          790       800       810       820       830       840
            *         *         *         *         *         *
CTG AAG GAA TCA GAC AAA GAC AGA AGA CTG TCC GTA GAG ATC TGG GAT TGG GAC CTG ACC
Leu Lys Glu Ser Asp Lys Asp Arg Arg Leu Ser Val Glu Ile Trp Asp Trp Asp Leu Thr
          850       860       870       880       890       900
            *         *         *         *         *         *
AGC AGG AAT GAC TTC ATG GGA TCT CTG TCG TTT GGG ATT TCA GAA CTA CAG AAA GCC GGA
Ser Arg Asn Asp Phe Met Gly Ser Leu Ser Phe Gly Ile Ser Glu Leu Gln Lys Ala Gly
          910       920       930       940       950       960
            *         *         *         *         *         *
GTG GAT GGC TGG TTC AAG TTA CTA AGC CAG GAA GAA GGC GAG TAC TTT AAT GTG CCG GTG
Val Asp Gly Trp Phe Lys Leu Leu Ser Gln Glu Glu Gly Glu Tyr Phe Asn Val Pro Val
          970       980       990      1000      1010      1020
            *         *         *         *         *         *
CCG CCG GAA GAA AGC GAG GGC AAT GAA GAG CTG CGG CAG AAG TTT GAG AGA GCC AAG ATT
Pro Pro Glu Glu Ser Glu Gly Asn Glu Glu Leu Arg Gln Lys Phe Glu Arg Ala Lys Ile
         1030      1040      1050      1060      1070      1080
            *         *         *         *         *         *
GGC CAA GGT ACC AAG GCT CCA GAA GAA AAG ACA GCG AAC ACT ATA TCC AAA TTT GAC AAC
Gly Gln Gly Thr Lys Ala Pro Glu Glu Lys Thr Ala Asn Thr Ile Ser Lys Phe Asp Asn
         1090      1100      1110      1120      1130      1140
            *         *         *         *         *         *
AAT GGC AAC AGG GAC CGG ATG AAA CTG ACC GAT TTT AAC TTC CTG ATG GTG CTG GGG AAA
Asn Gly Asn Arg Asp Arg Met Lys Leu Thr Asp Phe Asn Phe Leu Met Val Leu Gly Lys
         1150      1160      1170      1180      1190      1200
            *         *         *         *         *         *
GGC AGC TTT GGC AAG GTC ATG CTC TCA GAG CGG AAG GGT ACA GAT GAA CTC TAT GCC GTG
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FIG.1. CONT.

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Gly Ser Phe Gly Lys Val Met Leu Ser Glu Arg Lys Gly Thr Asp Glu Leu Tyr Ala Val
            1210           1220           1230           1240           1250           1260
               *              *              *              *              *              *
AAG ATC CTG AAG AAA GAT GTG GTG ATC CAA GAT GAC GAT GTG GAG TGC ACA ATG GTG GAG
Lys Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp Asp Val Glu Cys Thr Met Val Glu
            1270           1280           1290           1300           1310           1320
               *              *              *              *              *              *
AAG AGG GTG CTG GCC CTG CCT GGG AAG CCC CCA TTC CTG ACT CAG CTC CAT TCC TGC TTC
Lys Arg Val Leu Ala Leu Pro Gly Lys Pro Pro Phe Leu Thr Gln Leu His Ser Cys Phe
            1330           1340           1350           1360           1370           1380
               *              *              *              *              *              *
CAG ACC ATG GAC CGC CTC TAC TTT GTG ATG GAG TAT GTG AAC GGG GGC GAC CTC ATG TAC
Gln Thr Met Asp Arg Leu Tyr Phe Val Met Glu Tyr Val Asn Gly Gly Asp Leu Met Tyr
            1390           1400           1410           1420           1430           1440
               *              *              *              *              *              *
CAC ATC CAA CAA GTT GGC CGT TTC AAG GAG CCC CAT GCT GTA TTT TAC GCT GCA GAG ATT
His Ile Gln Gln Val Gly Arg Phe Lys Glu Pro His Ala Val Phe Tyr Ala Ala Glu Ile
            1450           1460           1470           1480           1490           1500
               *              *              *              *              *              *
GCC ATC GGT CTT TTC TTC TTG CAG AGC AAG GGC ATC ATT TAC CGT GAC CTG AAA CTT GAC
Ala Ile Gly Leu Phe Phe Leu Gln Ser Lys Gly Ile Ile Tyr Arg Asp Leu Lys Leu Asp
            1510           1520           1530           1540           1550           1560
               *              *              *              *              *              *
AAC GTG ATG CTG GAT TCC GAG GGG CAC ATC AAA ATC GCT GAC TTT GGC ATG TGT AAA GAG
Asn Val Met Leu Asp Ser Glu Gly His Ile Lys Ile Ala Asp Phe Gly Met Cys Lys Glu
            1570           1580           1590           1600           1610           1620
               *              *              *              *              *              *
AAT ATC TGG GAT GGG GTG ACA ACC AAG ACA TTC TGT GGC ACT CCA GAC TAC ATT GCC CCA
Asn Ile Trp Asp Gly Val Thr Thr Lys Thr Phe Cys Gly Thr Pro Asp Tyr Ile Ala Pro
            1630           1640           1650           1660           1670           1680
               *              *              *              *              *              *
GAG ATC ATT GCT TAT CAG CCC TAC GGA AAG TCT GTG GAC TGG TGG GCG TTT GGA GTC CTG
Glu Ile Ile Ala Tyr Gln Pro Tyr Gly Lys Ser Val Asp Trp Trp Ala Phe Gly Val Leu
            1690           1700           1710           1720           1730           1740
               *              *              *              *              *              *
CTG TAT GAA ATG TTG GCT GGC CAG GCA CCT TTT GAA GGG GAG GAT GAG GAT GAA CTC TTC
Leu Tyr Glu Met Leu Ala Gly Gln Ala Pro Phe Glu Gly Glu Asp Glu Asp Glu Leu Phe
            1750           1760           1770           1780           1790           1800
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FIG. 1. CONT.

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                *               *               *               *               *               *
CAG TCA ATC ATG GAG CAC AAC GTG GCG TAT CCC AAG TCC ATG TCT AAG GAA GCT GTG GCA
Gln Ser Ile Met Glu His Asn Val Ala Tyr Pro Lys Ser Met Ser Lys Glu Ala Val Ala
             1810            1820            1830            1840            1850            1860
                *               *               *               *               *               *
ATC TGC AAA GGG CTA ATG ACC AAA CAC CCA GGC AAG CGC CTG GGT TGT GGG CCT GAA GGG
Ile Cys Lys Gly Leu Met Thr Lys His Pro Gly Lys Arg Leu Gly Cys Gly Pro Glu Gly
             1870            1880            1890            1900            1910            1920
                *               *               *               *               *               *
GAA CGA GAC ATT AAG GAG CAT GCA TTT TTC CGG TAT ATC GAC TGG GAG AAA CTC GAA CGC
Glu Arg Asp Ile Lys Glu His Ala Phe Phe Arg Tyr Ile Asp Trp Glu Lys Leu Glu Arg
             1930            1940            1950            1960            1970            1980
                *               *               *               *               *               *
AAG GAG ATT CAG CCA CCT TAT AAA CCA AAA GCT AGA GAC AAG CGA GAC ACC TCC AAC TTC
Lys Glu Ile Gln Pro Pro Tyr Lys Pro Lys Ala Arg Asp Lys Arg Asp Thr Ser Asn Phe
             1990            2000            2010            2020            2030            2040
                *               *               *               *               *               *
GAC AAA GAG TTC ACC AGG CAG CCT GTG GAA CTG ACT CCC ACT GAC AAA CTC TTC ATC ATG
Asp Lys Glu Phe Thr Arg Gln Pro Val Glu Leu Thr Pro Thr Asp Lys Leu Phe Ile Met
             2050            2060            2070            2080            2090            2100
                *               *               *               *               *               *
AAC TTG GAC CAA AAT GAA TTT GCT GGC TTC TCG TAT ACT AAC CCA GAG TTT GTC ATT AAT
Asn Leu Asp Gln Asn Glu Phe Ala Gly Phe Ser Tyr Thr Asn Pro Glu Phe Val Ile Asn
             2110            2120            2130            2140            2150            2160
                *               *               *               *               *               *
GTG TAG GTG AAT GCA GAT TCC ATC GCT GAG CCT GTG TGT AAG GCT GCA GCG TGA ATG TCT
Val ---
             2170            2180            2190            2200            2210            2220
                *               *               *               *               *               *
ATT ATC AAT TCC AGT CTT CCA GGA TTC ATG GTG CCT CTG TTG GCA TCC GTC ATG TGG AGA

             2230            2240            2250            2260            2270            2280
                *               *               *               *               *               *
GCT TGT CTT AGA GGG CTT TTC TTT GTA TGT ATA GCT TGC TAG TTT GTT TTC TAC ATT TCA

             2290            2300            2310            2320            2330            2340
                *               *               *               *               *               *
AAA TGT TTA GTT TAG AAT AAG TGC ATT GCC CAC TGA TAG AGG TAC AAT TTT CCA GAC TTC
```

FIG. 1. CONT.

```
                2350           2360          2370            2380           2390           2400
                   *              *             *               *              *              *
CAG AAA CTC ATC CAA TGA ACC AAC AGT GTC AAA ACT TAA CTG TGT CCG ATA CCA AAA TGC

                2410           2420          2430            2440           2450           2460
                   *              *             *               *              *              *
TTC AGT ATT TGT AAT TTT TAA AGT CAG ATG CTG ATG TTC CTG GTC AAA GTT TTT ACA GTT

                2470           2480          2490            2500           2510           2520
                   *              *             *               *              *              *
ACT CTC GAA TAT CTC CTT TGA ATG CTA CCT AAG CAT GAC CGG TAT TTT TAA AAG TTG TGA

                2530           2540          2550            2560           2570           2580
                   *              *             *               *              *              *
GTA AGC TTT GCA GTT ACT GTG AAC TCT TGT CTC TTG GAG GAA CTT TTT GTT TAA GAA TTG

                2590           2600
                   *              *
GTA TGA TTA AAC TGA ATT CT
```

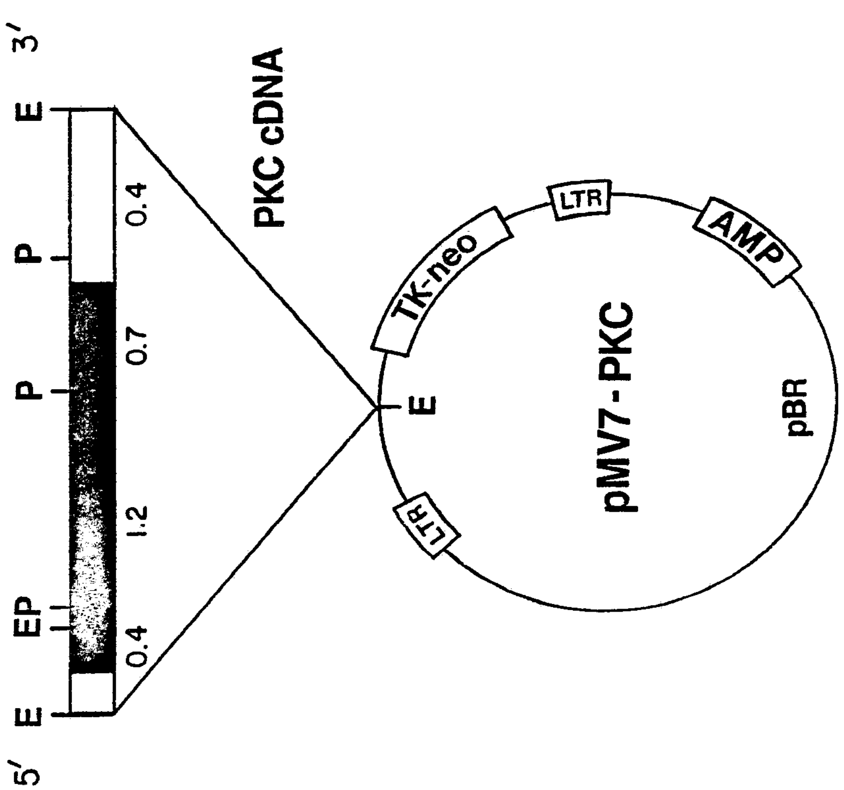

5′
3′
E
EP
P
P
E
0.4
1.2
0.7
0.4
PKC cDNA
TK-neo
LTR
AMP
LTR
E
pMV7-PKC
pBR

FIG. 1. B.

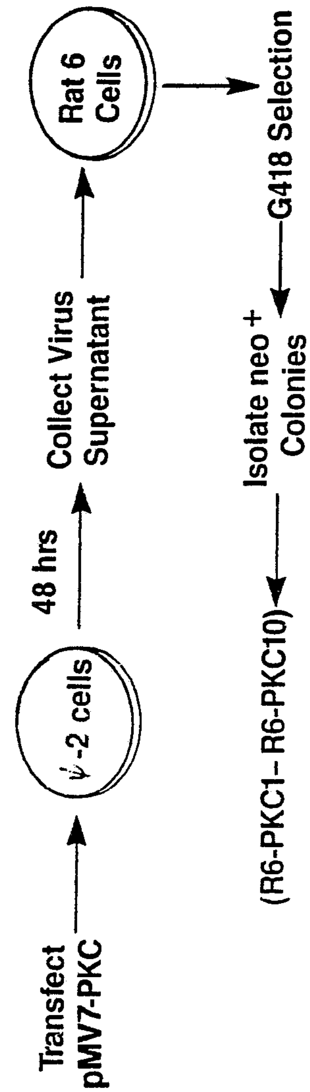

Transfect
pMV7-PKC
ψ-2 cells
48 hrs
Collect Virus
Supernatant
Rat 6
Cells
G418 Selection
Isolate neo+
Colonies
(R6-PKC1– R6-PKC10)

FIG. 1. C.

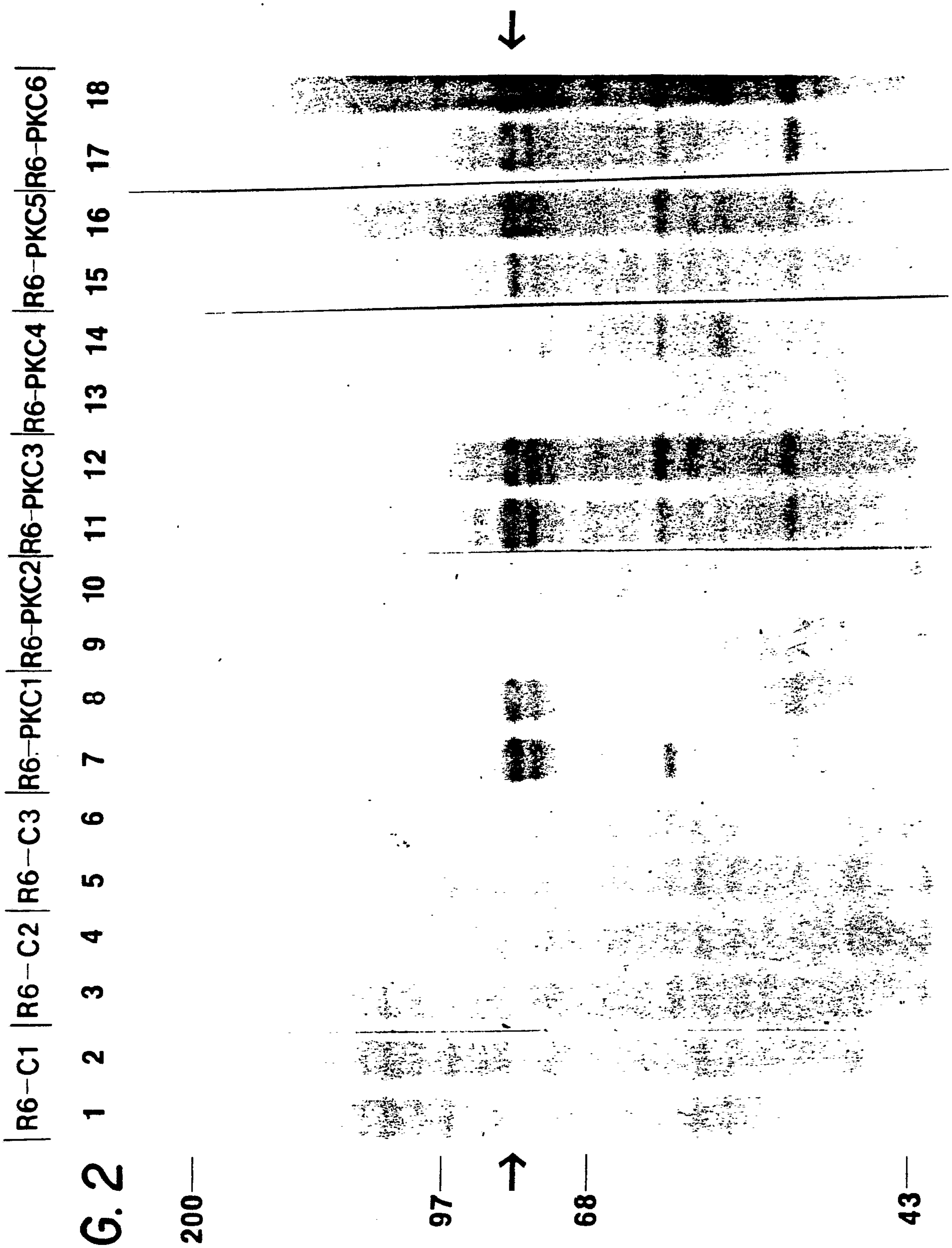
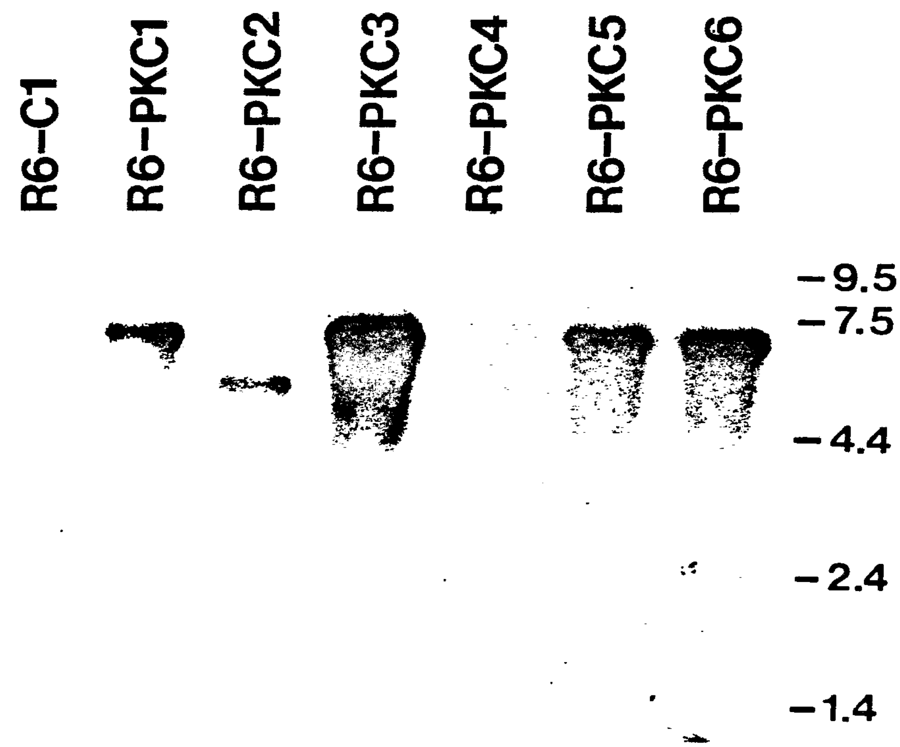

R6–C1
R6–C2
R6–C3
R6–PKC1
R6–PKC2
R6–PKC3
R6–PKC4
R6–PKC5
R6–PKC6
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
200
97
68
43

FIG. 2

FIG. 3


R6-C1
R6-PKC1
R6-PKC2
R6-PKC3
R6-PKC4
R6-PKC5
R6-PKC6
-9.5
-7.5
-4.4
-2.4
-1.4

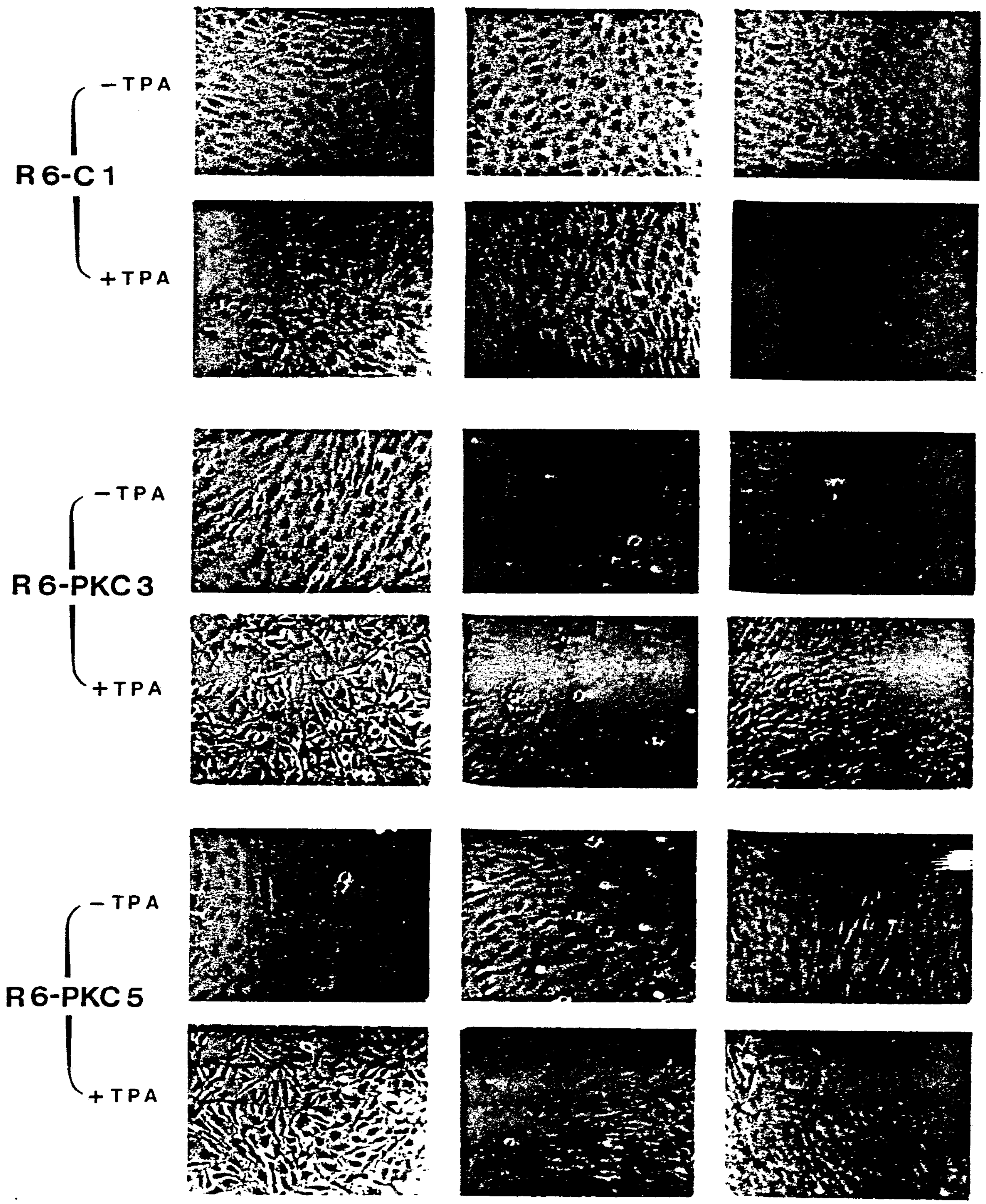
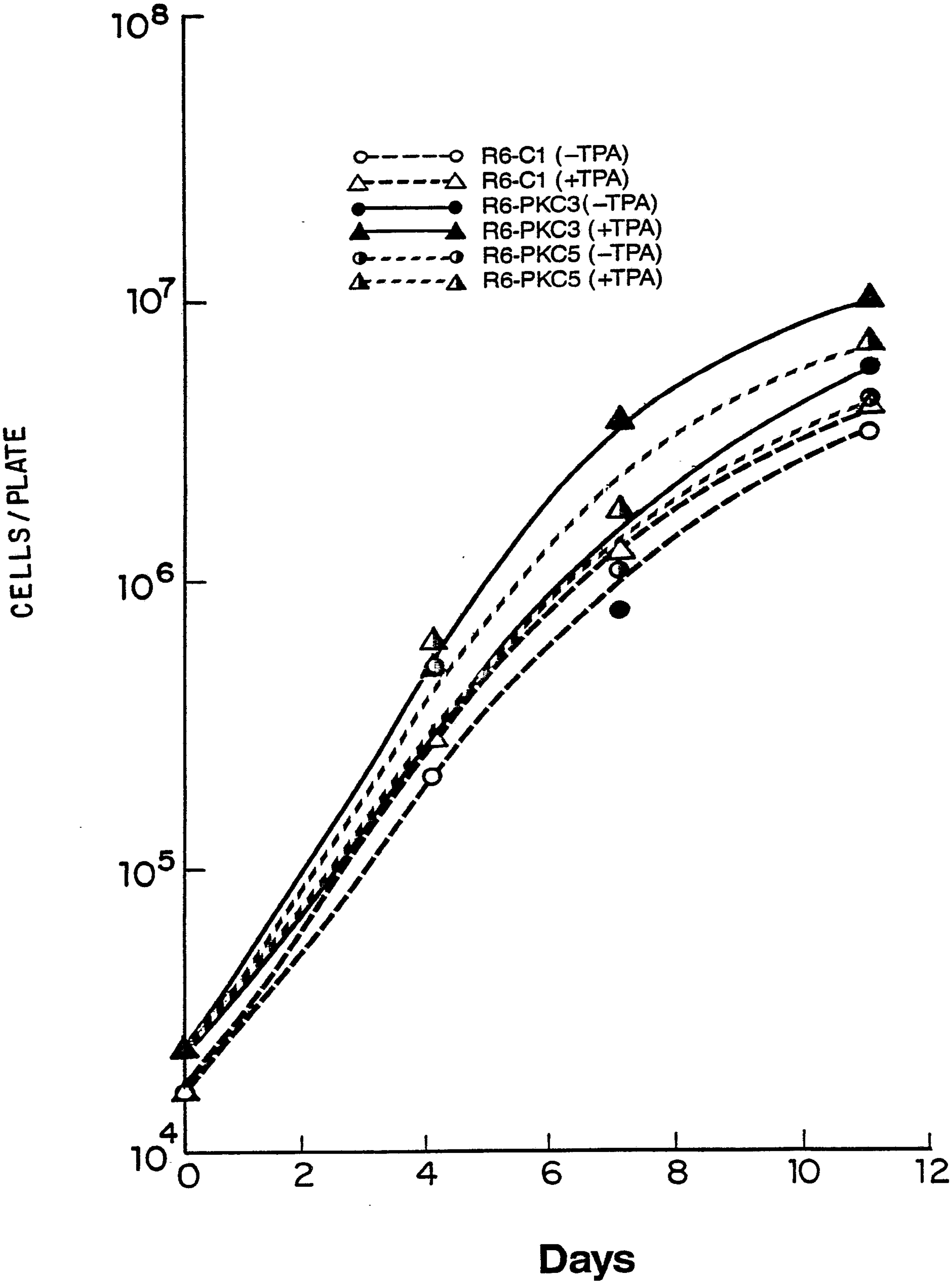


FIG.4

FIG. 5.



10^8
10^7
10^6
10^5
10^4
CELLS/PLATE
R6-C1 (−TPA)
R6-C1 (+TPA)
R6-PKC3(−TPA)
R6-PKC3 (+TPA)
R6-PKC5 (−TPA)
R6-PKC5 (+TPA)
0
2
4
6
8
10
12
Days

FIG. 6

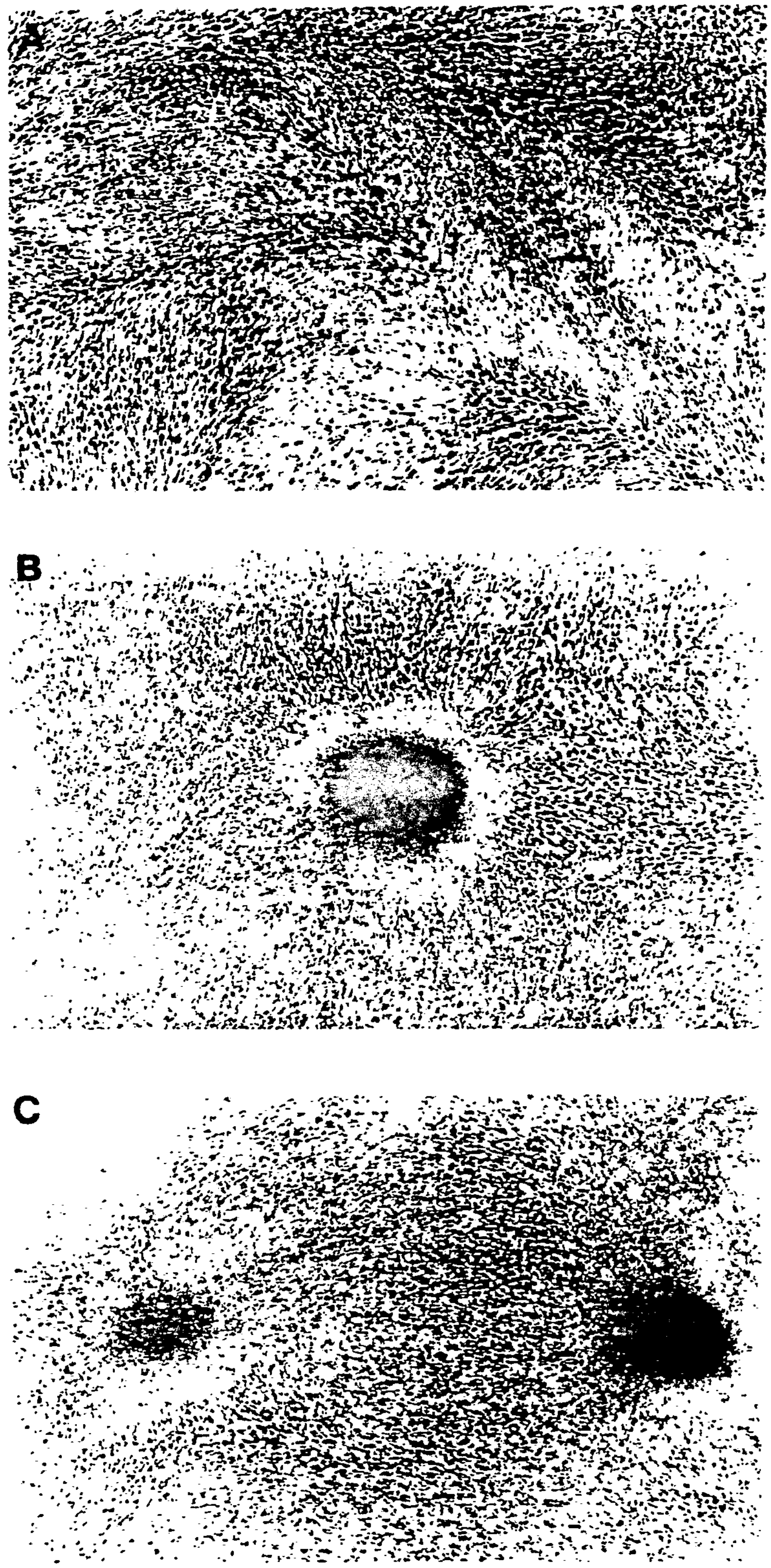
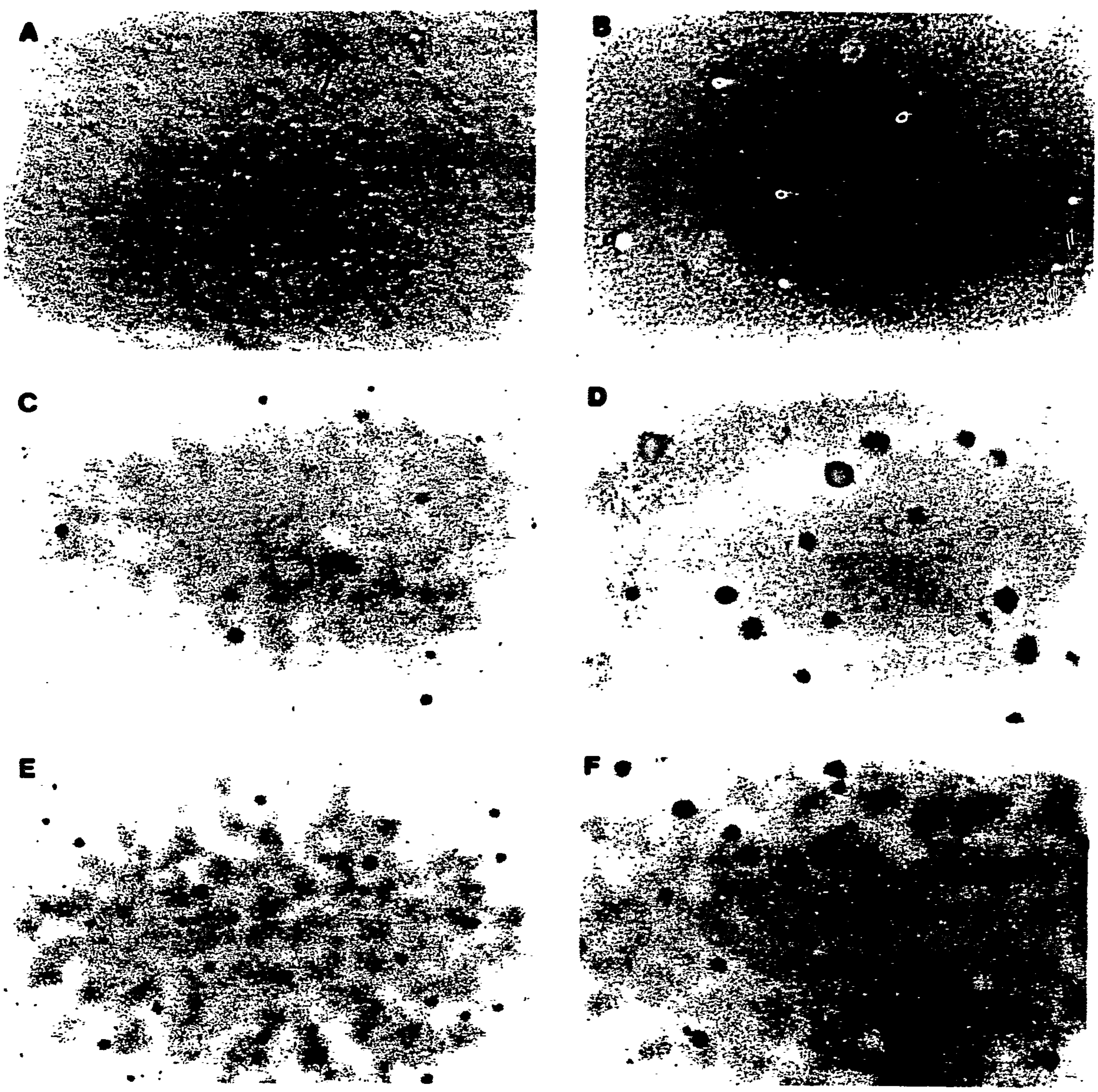


FIG. 7



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/00462

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 1/02

U.S. Cl.: 435/29

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/29

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A X	US, A, 4,480,038 (CHENG) 30 October 1984 (see col. 2, lines 32-40 and 60-68)	1-20 and 24 21-23
A	US, A, 4,532,204 (CRESPI) 30 July 1985	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 April 1989	12 JUL 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Sam Rosen SAM ROSEN